CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Roehampton University Research Repository

Elsevier Editorial System(tm) for Experimental Cell Research
Manuscript Draft

Manuscript Number: ECR-10-81R1

Title: Transcription factor KLF7 regulates differentiation of neuroectodermal and mesodermal cell lineages

Article Type: Research Article

Keywords: BLBP/FABP7; Map2; neuritogenesis; cardiomyogenesis; adipogenesis; osteogenesis

Corresponding Author: Dr. Massimiliano Caiazzo, Ph.D.

Corresponding Author's Institution: Institute of Genetics and Biophysics

First Author: Massimiliano Caiazzo, Ph.D.

Order of Authors: Massimiliano Caiazzo, Ph.D.; Luca Colucci-D'Amato, MD PhD; Maria T Esposito, PhD; Silvia Parisi, PhD; Stefano Stifani, PhD; Francesco Ramirez, PhD; Umberto di Porzio, MD/PhD

Abstract: Previous gene targeting studies in mice have implicated the nuclear protein Krüppel-like factor 7 (KLF7) in nervous system development while cell culture assays have documented its involvement in cell cycle regulation. By employing short hairpin RNA (shRNA)-mediated gene silencing, here we demonstrate here that murine Klf7 gene expression is required for in vitro differentiation of neuroectodermal and mesodermal cells. Specifically, we show a correlation of Klf7 silencing with down-regulation of the neuronal marker microtubule-associated protein 2 (Map2) and the nerve growth factor (NGF) tyrosine kinase receptor A (TrkA) using the PC12 neuronal cell line. Similarly, KLF7 inactivation in Klf7-null mice decreases the expression of the neurogenic marker brain lipid-binding protein/fatty acid-binding protein 7 (BLBP/FABP7) in neural stem cells (NSCs). We also report that Klf7 silencing is detrimental to neuronal and cardiomyocytic differentiation of embryonic stem cells (ESCs), in addition to altering the adipogenic and osteogenic potential of mouse embryonic fibroblasts (MEFs). Finally, our results suggest that genes that are key for self-renewal of undifferentiated ESCs repress Klf7 expression in ESCs. Together with previous findings, these results provide evidence that KLF7 has a broad spectrum of regulatory functions, which reflect the discrete cellular and molecular contexts in which this transcription factor operates.

Naples May 17th, 2010
Prof Urban Lendhal
Editor in Chief
Experimental Cell research

Dear Prof. Lendhal,
We have accepted the suggestions from the Experimental Cell Research reviewers on our article "Transcription factor KLF7 regulates differentiation of neuroectodermal and mesodermal cell lineages", which we submitted on the 5th February 2010, and are grateful for the clarifications they propose. We have modified the text accordingly.

In order to address all the issues raised, we have performed additional experiments and added two new figures (now Fig. 2 and Fig. 4) to the paper, as well as modified three other figures (now Fig. 1, Fig. 7 and Fig. 8).

We hope think that we have addressed all the issues raised by the reviewers and we hope that you will find this revised version of our manuscript acceptable for publication.

Looking forward to hearing from you, I remain

Yours sincerely,

Massimiliano Caiazzo

In order to address all the issues raised, we have performed additional experiments and added two new figures (now Fig. 2 and Fig. 4) to the paper, as well as modified three other figures (now Fig. 1, Fig. 7 and Fig. 8).
The specific answers to each point are highlighted below:

Reviewer #1

We are happy to clarify the point raised by the reviewer #1. As asked we added in the Material and methods section the cell number used in control and experimental groups in MEFs experiments. In particular at page 8 (revised version, section MEFs adipogenic differentiation) we have modified the sentence: "*Same number of MEFs were plated, $20x10^4$ cells/cm^2, in control and Klf7-silenced samples*" and at page 8 (section MEFs osteogenic differentiation) we have modified the sentence: *"Same number of MEFs were plated $10x10^4$ cells/cm^2, in control and Klf7-silenced samples*".

Reviewer #2

1) As asked by reviewer #2, we clarified in Fig. 2B caption (now Fig. 3B, page 32), that the western blot analysis showed represents KLF7 down-regulation by shRNA in undifferentiated ESCs. We also added in the Results section, at page 15, the sentence: "*We then performed Klf7 gene silencing experiments. We first showed in Fig. 3B a good efficiency of KLF7 down-regulation by shRNA in undifferentiated ESCs*".

2) Regarding Fig. 2C (now Fig. 3C), neurite formation at day 10 is comparable between control and *Klf7* silenced ESCs. Thus we added in the Results section (page 15) the sentence: "*At day 10 following differentiation neurite formation recovers in Klf7 silenced ESCs and becomes comparable to the control samples (data not shown).*" and modified the Fig. 3C caption (page 32): "*Neurite outgrowth is delayed in shKlf7-ESCs after differentiation as observed at day 8, whereas (data not shown) no differences were observed at day 10*". In addition as asked by the reviewer #2 we added a molecular analysis to characterize the transition from a proliferative to a differentiated stage. The results of these experiments are shown in a new figure (Fig. 4 revised version), and were added in the Results section (page 15): "*Finally, to assess the involvement of KLF7 in the transition from proliferation to the differentiation, we carried out a molecular analysis using proliferation and neural markers in control and Klf7-silenced ESCs during differentiation. With regard to the proliferation markers, in Klf7-silenced ESCs, we found a transient down-regulation of $p21^{waf/cip}$ transcripts between days 0 and 4 and an increase of Ki67 mRNA at day 0. Neural differentiation was assessed by analyzing the expression of neuronal (βIII tubulin), glial (Gfap) and oligodendroglial (Ng2) markers. We found that only βIII tubulin transcripts were transiently decreased in Klf7 silenced samples (Fig. 4)*".

3) We did not observe any difference in neurite formation between wt and *Klf7* -/ - NSCs. *Klf7* expression differs throughout mouse embryonic development (Laub et al., 2001) and is reduced at birth. Since NSCs used for these experiments were obtained from newborn mice, a likely explanation lies upon a different role exerted by KLF7 during early phase of development versus newborn stage (see Discussion section at page 20).

4) As asked by the reviewer #2 we have commented in the Discussion section (page 20) the finding that although a reduction of BLBP/ FABP7 is observed in *Klf7* -/ - NSCs these cells normally differentiated in the three neural lineages. Thus we added the sentence: "*These data are consistent with previous findings that Blbp/Fabp7 -/- mice display normal brain differentiation as observed by morphological and immunohistological analyses [38]*".

5) We have clarified that we did not observe any delay in the appearance of beating activity in *Klf7*-silenced (sh*Klf7*) ESCs compared to the control, moreover we did not observe any increase in the number of beating embryoid bodies derived from sh*Klf7*-ESCs at day 10 or later. Thus we added the sentence in the Results section (page 17): "*More interestingly, we observed a dramatic decrease in the number of beating cardiomyocytes derived from shKlf7-ESCs when compared to control cells at day 8. We did not observe a delay in the appearance of beating activity in shKlf7-ESCs compared to the control. We did not observe further decrease in the number of beating embryoid bodies derived from shKlf7-ESCs at day 10 or later (Fig. 6B)*".

6) We modified the following sentence in the Results section (page 18): "This *analysis did not show any significant difference in undifferentiated/proliferating cells (Fig. 8A), possibly because Klf7 transcript levels are too low under these culture conditions. Therefore, we analyzed Klf7 transcripts level after 13 days following induction of ESCs differentiation by serum deprivation, a process that results in increased Klf7 expression (Fig. 8A).*" clarifying the issue raised by the reviewer #2 about the stage of the performed analysis and the expression of *Oct4* and *Nanog* genes during wt ESCs differentiation. Thus we performed a real time RT-PCR experiment showing the expression of *Oct4* and *Nanog* transcripts during wt ESCs differentiation. In agreement with previous findings by others our data show that the two transcripts strongly decrease between day 4 and day 8 following differentiation, in addition we show that subsequently they recover with a modest increase between day 11 and day 13.

Reviewer #3

1) We addressed the issue raised by reviewer #3 about experiments performed in PC12 cells. As asked, we quantitated the reduction of KLF7 protein in sh*Klf7* PC12

cells, and found 84% of decrease. Thus we modified Fig. 1 and we also added this new information in the Results section (page 13): "*Levels of Klf7 transcripts and KLF7 protein (84% decrease) were strongly reduced in proliferating cells stably transfected with the construct expressing a Klf7 shRNA but not with a non-silencing control construct (Fig. 1B and 1C)".* Then we quantitated the reduction of TRKA protein expression in sh*Klf7*-PC12 cells and we modified the results section accordingly (page 13) : "*Klf7 silencing was associated with a significant down-regulation of the NGF receptor TrkA transcripts and protein (Fig. 1B and 1C).*" Moreover we showed and quantitated the decreased differentiation of *Klf7*-silenced PC12 cell upon NGF treatment (Fig. 1D) as reported in the Results section (page 14): "*Our study showed that induction of neuronal differentiation by NGF treatment in Klf7-silenced PC12 cells resulted in a strong decrease of neurite outgrowth compared to controls (Fig. 1D)*". Finally we added a new figure (Fig. 2, revised version) showing that NGF-dependent gene expression (*Vgf, Scg10*) in sh*Klf7*-PC12 cells was significantly affected. In the same figure we showed that the cell cycle regulator $p21^{waf/cip}$ transcripts strongly increased following NGF stimulation in control cells, whereas such increase was significantly less marked in *Klf7* silenced cells. Thus we modified the Results section accordingly (page 14): "*To corroborate these findings we analyzed in PC12 stimulated cultures NGF-dependent gene expression showing that both Vgf and Scg10 transcripts were less expressed in silenced as compared to control cells (Fig. 2). Moreover since NGF-induced PC12 differentiation is paralleled by cell cycle exit we also evaluated the expression of* $p21^{waf/cip}$ *cell cycle regulator. We found that in Klf7 silenced PC12 cells,* $p21^{waf/cip}$ *was strongly affected when compared to control*." Accordingly we also modified the discussion section (page 19): "*It is worth noting that the silencing of Klf7 in PC12 cells not only decreases TRKA protein expression, but also down-regulates NGF-dependent signaling as showed by Vgf and Scg10 expression. The latter are two genes known to be altered when NGF signaling is affected [33,34].*"

2) We addressed the issue raised by reviewer #3 concerning experiments performed in ESCs. As asked, we added a new figure (Fig. 4, revised version) showing a time course of early differentiation markers (*βIII tubulin*, *Gfap*, *Ng2*) and cell cycle markers ($p21^{waf/cip}$, *Ki67*) along differentiation either in control and *Klf7* silenced ESCs. The text has been modified accordingly in the Results section (page 15): "*Finally, we carried out a molecular analysis of proliferation and neural markers in control and Klf7 silenced ESCs during differentiation. Regarding the proliferation markers, in Klf7 silenced ESCs, we found a transient down-regulation of* $p21^{waf/cip}$ *and Ki67 transcripts between days 0 and 4 and an increase of Ki67 mRNA at day 0. Neural differentiation was assessed by analyzing the expression of neuronal (βIII tubulin) glial (Gfap) and oligodendroglial (Ng2) markers. We found that only βIII tubulin transcripts were transiently decreased in Klf7 silenced samples (Fig. 4).*"
Finally, in the paragraph **KLF7 is important for neuronal differentiation in PC12 neuroepithelial cells and ESCs,** in now page 15 the phrase "*there was a significant increase in Klf7 gene expression during E14Tg2A neuronal differentiation, which reaches*

a plateau at 10d (Fig. 3A), similar to that shown in Fig.1 for PC12 cells)." has been modified into "*there was a significant increase in Klf7 gene expression during E14Tg2A neuronal differentiation, which reaches a plateau at 10d (Fig. 3A)*", as requested.

3) According to reviewer #3 suggestion, we reformulated the sentence linking BLBP/ FABP7 down-regulation to neurogenic induction as follows (Discussion section section, page 20): "*Therefore, although we did not find major alterations in neural phenotypes differentiated from Klf7 -/- NSCs, our finding show that the number of cells expressing BLBP/FABP7is decreased suggests that a subtle alteration in the phenotypic differentiation may occur in the absence of KLF7. A deeper and more extensive analysis is needed to resolve this issue.*"

4) As asked by reviewer #3 the corresponding *Klf7* western blot for cardiomyocytes differentiation is shown in Fig. 3B (revised version), since cardiomyocytes and neuronal differentiation were performed on the same *Klf7*-silenced ESCs clones. Regarding the number of embryoid bodies, they were counted for beating as stated in the Material and methods section (page 6): "*48 EBs were counted in three different experiments in each experimental condition*".

5) The beating cardiomyocytes counted at day 8 of differentiation was decreased when compared to normal controls. No further decrease was observed at day 10 between control and *Klf7*-silenced ESCs. Accordingly, we added the following sentence in the results section (page 17): "*More interestingly, we observed a dramatic decrease in the number of beating cardiomyocytes derived from shKlf7-ESCs when compared to control cells at day 8. We did not observe a delay in the appearance of beating activity in shKlf7-ESCs compared to the control. We did not observe further decrease in the number of beating embryoid bodies derived from shKlf7-ESCs at day 10 or later (Fig. 6B)*". Following reviewer #3 suggestion about Fig. 5 (now Fig. 7) we changed it accordingly.

6) As asked by the reviewer #3, we analyzed *Klf7* expression upon overexpression of either *Oct4* or *Nanog* in ESCs. The results showed in Fig. 8 are consistent with the postulated link between *Klf7* expression and the ESCs self-renewal master genes *Oct4* and *Nanog*. Results have been changed accordingly (page 18): "*Moreover we analyzed Klf7 transcripts upon overexpression of either Oct4 or Nanog genes in ESCs. The results of the qRT-PCR showed a significant decrease of Klf7 expression (Fig. 8B).*". The concern raised about the effect of *Klf7* overexpression in ESCs, has been already addressed by Ivanova et al. (2006). Indeed they found that *Klf7* overexpression in ESCs leads to differentiated morphology, loss of alkaline phosphatase activity and *Nanog* down-regulation. Altogether these data are consistent with a role of *Klf7* in processes of ESCs proliferation and differentiation. We accordingly modified the Discussion section (page 21): "*Moreover either Oct4 or Nanog overexpression leads to Klf7 transcripts down-regulation. On the other hand, as shown by Ivanova et al., Klf7 overexpression results in*

ESCs differentiation, loss of alkaline phosphatase activity and Nanog down-regulation. Taken together these data point towards a role of KLF7 in ESCs proliferation and differentiation mechanisms.".

7) Finally, regarding technical question, we used at least three independent replicates for RT-PCR and western blot quantitation. All the shown experiments were performed at least twice as indicated in the Material and methods section (page 12).

*Revised Manuscript
Click here to view linked References

Transcription factor KLF7 regulates differentiation of neuroectodermal and mesodermal cell lineages

Massimiliano Caiazzo[a,b,*], Luca Colucci-D'Amato[a,c], Maria T. Esposito[d,1], Silvia Parisi[d], Stefano Stifani[e], Francesco Ramirez[f], Umberto di Porzio[a]

[a]Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, 80131 Naples, Italy

[b]Istituto di diagnosi e cura "Hermitage Capodimonte", 80131 Naples, Italy

[c]Department of Life Science, "Seconda Università di Napoli", 81100 Caserta, Italy

[d]CEINGE Biotecnologie Avanzate, 80145 Naples , Italy

[e]Centre for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, Quebec H3A 2B4, Canada

[f]Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY 10029, USA

[1] present address Dept. Hematology, King's College London, UK

[*]Corresponding author. Tel.: +39 081 6132363; Fax: +39 081 6132350

E-mail addresses: caiazzo@igb.cnr.it (M. Caiazzo), luca.colucci@unina2.it (L. Colucci-D'amato), maria_teresa.esposito@kcl.ac.uk (M.T. Esposito), parisi@ceinge.unina.it (S. Parisi), stefano.stifani@mcgill.ca (S. Stifani), francesco.ramirez@mssm.edu (F. Ramirez), diporzio@igb.cnr.it (U. di Porzio).

ABSTRACT

Previous gene targeting studies in mice have implicated the nuclear protein Krüppel-like factor 7 (KLF7) in nervous system development while cell culture assays have documented its involvement in cell cycle regulation. By employing short hairpin RNA (shRNA)-mediated gene silencing, here we demonstrate here that murine *Klf7* gene expression is required for *in vitro* differentiation of neuroectodermal and mesodermal cells. Specifically, we show a correlation of *Klf7* silencing with down-regulation of the neuronal marker microtubule-associated protein 2 (*Map2*) and the nerve growth factor (NGF) tyrosine kinase receptor A (*TrkA*) using the PC12 neuronal cell line. Similarly, KLF7 inactivation in *Klf7*-null mice decreases the expression of the neurogenic marker brain lipid-binding protein/ fatty acid-binding protein 7 (BLBP/ FABP7) in neural stem cells (NSCs). We also report that *Klf7* silencing is detrimental to neuronal and cardiomyocytic differentiation of embryonic stem cells (ESCs), in addition to altering the adipogenic and osteogenic potential of mouse embryonic fibroblasts (MEFs). Finally, our results suggest that genes that are key for self-renewal of undifferentiated ESCs repress *Klf7* expression in ESCs. Together with previous findings, these results provide evidence that KLF7 has a broad spectrum of regulatory functions, which reflect the discrete cellular and molecular contexts in which this transcription factor operates.

Keywords: BLBP/ FABP7; *Map2*; neuritogenesis; cardiomyogenesis; adipogenesis; osteogenesis.

ABBREVIATIONS: bFGF, basic fibroblast growth factor; BLBP/ FABP7, brain lipid-binding protein/ fatty acid-binding protein 7; CNS, central nervous system; E, embryonic day; EBs, embryoid bodies; EGF, epidermal growth factor; ESCs, embryonic stem cells; FBS, fetal bovine serum; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HS, horse serum; KLF7, Krüppel-like factor 7; *Klf7* -/ -, *Klf7*-homozygous null mutant; *Map2*, microtubule-associated protein 2; MEFs, mouse embryonic fibroblasts; NGF, nerve growth factor; NS, nervous system; NSCs, neural stem cells; *Oct4*, octamer-binding transcription factor 4; P, postnatal day; qRT-PCR, quantitative real-time RT-PCR; RNAi, RNA interference; RT, room temperature; SGZ, striatal germinal zone; shRNA, short hairpin RNA; *TrkA*, tyrosine kinase receptor A; wt, wild type.

INTRODUCTION

Krüppel-like Factor 7 (KLF7) belongs to the relatively large KLF family of

transcription factors that regulate a variety of cellular programs during embryonic development and in the adult organism [1,2]. We have previously identified KLF7 through a PCR-based screen and have characterized this protein as a key regulator of neurogenesis and cell cycle progression. More specifically, KLF7 overexpression causes cell cycle arrest by up-regulating two well-known cell cycle regulators, $p21^{waf/cip}$ and $p27^{kip1}$ [3]. *In situ* hybridization analyses showed that although *Klf7* is almost ubiquitously expressed in the adult mouse, its expression is mainly restricted to the developing nervous system (NS) in the embryo [3]. In agreement with the latter observation, *Klf7* inactivation in *Klf7*-homozygous null mutant (*Klf7* -/ -) mice causes severe abnormalities in the NS, including hypoplastic olfactory bulbs, impaired dendritic arborization in the cortex and hippocampus, and alterations of nociceptive sensory and sympathetic neurons [4,5].

Previous studies have also demonstrated that KLF7 overexpression inhibits the differentiation of both mouse 3T3-L1 preadipocytes and human preadipocytes [6,7]. Additionally, high-throughput analyses in embryonic stem cells (ESCs) suggested that KLF7 is potentially implicated in the regulation of ESCs self-renewal [8,9]. These findings raise the possibility that KLF7 might also exert regulatory roles in other tissues, in addition to the NS. This possibility remains to be answered, however, mainly due to the neonatal lethality of *Klf7* -/ - mice. The present study aimed at testing the involvement of KLF7 in cell differentiation using a genetic approach in various cell types. Our findings

indicate that KLF7 influences various aspects of neuronal, cardiomyocytic, adipocytic and osteocytic cell differentiation, as well as the maintenance of the stem cell phenotype. These findings improve our understanding of the multiple roles of KLF proteins in organ formation and function, in addition to confirming their involvement in stem cell self-renewal and differentiation.

MATERIAL AND METHODS

Cell cultures

PC12 cells. PC12, a cell line derived from a pheochromocytoma of the rat adrenal medulla, cells were grown in RPMI medium (Invitrogen, Milan, Italy), with 10% horse serum (HS, Invitrogen), 5% fetal bovine serum (FBS, Invitrogen) and 100U/ ml penicillin/ streptomycin (Invitrogen). To induce neuronal differentiation cells were previously starved for 24 hr before nerve growth factor (NGF, Alexis Biochemicals, San Diego, CA, USA)] 100 ng/ ml was added to the medium. Neurite formation was analyzed 7 days after differentiation induction. Total neurite length was performed using QWin software (Leica Microsystems, Milan, Italy) on five fields from three replicates for each condition.

ESCs. Undifferentiated E14Tg2A ESCs were grown without feeders onto 0.1%

gelatin-coated tissue dishes, in DMEM (Invitrogen) supplemented with 15% FBS (Hyclone, Logan, UT, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Milan, Italy), 1 mM sodium pyruvate (Invitrogen), 1X non essential aminoacids (Invitrogen), 2 mM glutamine (Invitrogen), 100 U/ ml penicillin/ streptomycin (Invitrogen) and 10^3 U/ ml leukemia inhibitory factor (Chemicon International, Temecula, CA, USA). ESCs were routinely passaged every 2 days, and the medium was changed on alternate days.

ESCs neuronal differentiation. 3 x 10^3 cells/ cm^2 ESCs were plated on 0.1% gelatin coated tissue culture in differentiation medium: Knockout-DMEM supplemented with 15% Knockout Serum Replacement (KnockOut™ SR, both from Invitrogen), 0.1 mM β-mercaptoethanol, 2 mM glutamine, 100 U/ ml penicillin/ streptomycin. Neurite outgrowth was analyzed 8 days after neuronal induction [10-13].

ESCs cardiomyogenic differentiation. 20 μl drops of ESCs (400 cells/ drop) in differentiation medium: [(DMEM (Invitrogen) supplemented with 15% FBS (Hyclone), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 1 mM sodium pyruvate (Invitrogen), 1X non essential aminoacids (Invitrogen), 2 mM glutamine (Invitrogen), 100U/ ml penicillin/ streptomycin (Invitrogen)] were placed on the lid of tissue culture dishes filled with PBS (Euroclone, Milan, Italy), and cultivated in hanging drops for 2 days. Two-days-old embryoid bodies (EBs)

were then collected in differentiation medium and transferred into 100-mm bacteriological Petri dishes. After further 3 days in culture, 5 days-old EBs were plated in the same medium onto 0.1% gelatin-coated tissue culture plates. Cardiomyocytic formation was assayed at 8 days in culture [14]. 48 EBs were counted in three different experiments in each experimental condition.

Neurosphere cultures. Using a stereoscopic light microscope under sterile conditions, postnatal day (P)0 brains were removed and overlying meninges and blood vessels were separated and discarded. The striatal germinal zone (SGZ) was dissected according to the coordinates reported in rodent brain atlas [15,16]. The tissue was transferred to 500 μl of DMEM/ F12 (Invitrogen) medium and mechanically dissociated into a cell suspension with a fire-polished Pasteur pipette. Cells were counted and cultured in suspension ($2,5x10^5$/ ml) with neurosphere medium [DMEM/ F12, B27 (Invitrogen), N2 (Invitrogen), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (both 20ng/ ml, Sigma-Aldrich)] in $25cm^2$ flasks with no substrate pre-treatment. Primary neurospheres formed after 4-5 days *in vitro* once every 7 days they were gently spun down (75g), mechanically dissociated, and the medium was changed. In order to maintain the primary neurosphere characteristics, all experiments were performed with cells passaged no more than 10 times.

Neurosphere neural differentiation. To evaluate neural differentiation potential,

neurospheres were dissociated and plated $5x10^4$ cells/ cm^2 on poly-D-lysine (15μg/ ml, Sigma-Aldrich) precoated plates in neurosphere medium. After 6 days in culture, cells were kept for additional 3 days without bFGF and EGF, and neuronal, astroglial and oligodendroglial differentiation was assessed by immunocytochemical analysis.

Neurosphere assays. To determine the self-renewal ability of secondary neurospheres, primary neurospheres were mechanically dissociated into single cells with a fire polished Pasteur pipette, counted and cultured at 500 cells/ 200 μl/ well in 96-well plates ultra-low attachment (Corning, Milan Italy) [17]. Each week, secondary neurospheres that had formed in each well were counted and passaged to a new single well. Twelve wells for both wild type (wt) and *Klf7* -/ - neurospheres were assayed during 4 weeks.

Mouse embryonic fibroblasts (MEFs) preparation. MEFs were prepared as previously described [18]. In order to maintain primary cell characteristics, MEF cells were not passaged more than six times in all experiments.

MEF adipogenic differentiation. Same number of MEFs were plated, $20x10^4$ cells/ cm^2, in control and *Klf7*-silenced samples and then cultured for two weeks in α-MEM (Invitrogen) supplemented with 10% FBS, 10% HS, 2 mM L-glutamine, 100 U/ ml penicillin/ streptomycin, 10^{-6} M dexamethasone (Sigma-

Aldrich), 5 μg/ ml insulin (Sigma-Aldrich), 0.5 μM 3-Isobutyl-1-methylxanthine (Sigma-Aldrich) and 50 μM indomethacin (Sigma-Aldrich). Culture medium was changed every 3-4 days [19].

MEF osteogenic differentiation. Same number of MEFs were plated $10x10^4$ cells/ cm^2 in control and *Klf7*-silenced samples and then cultured for 3 weeks in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 10 U/ ml penicillin -10 μg/ ml streptomycin,10^{-8} M dexamethasone, 0.05 mM ascorbic acid 2-phosphate (Sigma-Aldrich) and 10 mM β-glicerophosphate (Sigma-Aldrich). Culture medium was changed every 3-4 days [19].

Oil red O staining

MEF cells were washed with PBS and fixed in 4% paraformaldehyde for 10 minutes. After rinsing with distilled water, they were stained with 0.5% oil red O working solution prepared by vigorously mixing 3 parts of a stock solution (0.5% oil red O in isopropanol) (Sigma-Aldrich) with 2 parts of water for 5 minutes and filtering through a 0.4 μm filter. Excess staining was removed by rinsing twice with PBS. Cells were then counterstained with hematoxylin (Bio-Optica) for 3 minutes and then rinsed with distilled water [19]. Dye was extracted by isopropanol incubation for 15 minutes at room temperature (RT). Quantitative assessment was obtained by spectrophotometric analysis of absorbance of a five-fold dilution of the extracted dye at 540 nm.

Alizarin red S staining

MEF cells were rinsed with PBS and fixed in 4% paraformaldehyde for 1 hour; after rinsing with distilled water, cells were incubated with 2% alizarin red S (Sigma-Aldrich) pH 4.1 with gentle shaking for 10 minutes. Excess staining was removed by washing twice with PBS [19]. Dye was extracted by overnight incubation with 4M guanidine-HCl (Sigma-Aldrich) at RT. Absorbance at 490 nm of a five-fold dilution of the resulting supernatant was used for quantitative mineralization determination.

Cell transfections

All cell lines were transfected by lipofection using Lipofectamine 2000 transfection reagent (Invitrogen). All transfections were performed in triplicate samples. Transfected cells were selected with puromycin (Sigma-Aldrich) 2μg/ ml 48 hr after transfection. For each experimental condition three pools of resistant clones were generated and were kept separated in all quantitative real-time RT-PCR (qRT-PCR) and western blot experiments.

RNA interference (RNAi)

To silence *Klf7*, octamer-binding transcription factor 4 (*Oct4*) *or Nanog* gene expression we used short hairpin RNA (shRNA) expressing construct contained in the Expression Arrest™ mouse shRNA library (Open Biosystems, Huntsville,

AL, USA). In all RNAi experiments a short hairpin non-silencing construct was used as control.

Animals and dissections

Timed pregnant *Klf7*-heterozygous mutant mothers and postnatal (P)0 pups (Charles River Breeding Laboratories, Milan, Italy) were sacrificed in accordance with Society for Neuroscience guidelines and Italian law. The embryonic age was determined by considering the day of insemination (as confirmed by vaginal plug) as embryonic day (E)0. Embryos from E11.5 to E17.5 were quickly removed and placed in PBS, without calcium and magnesium, and supplemented with 33 mM glucose. All the tissues from embryos and pups were dissected according to the coordinates reported in rodent brain atlas [15,16] and processed for RNA extraction or cell cultures. All embryonic and postnatal tissues deriving from *Klf7*-heterozygous pregnant mothers were processed separately and a tail sample was processed for genotyping**.** Mice genotyping was performed as previously described [4].

RNA isolation and qRT-PCR analysis

RNA isolation and retro-transcription were performed as previously described [20,21]. qRT-PCR was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems). 1/ 50 of the reverse transcribed cDNA was amplified in a 25 μl reaction mixture containing 1X SYBR Green PCR Master Mix (Applied

Biosystems), and 0.4 μM each primer. The thermal profile consisted of 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. Gene expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression level. The primers used for the amplification were: *Gapdh* (forward 5'-GTATGACTCCACTCACGGCAAA-3', reverse 5'- TTCCCATTCTCGGCCTTG-3'), *Klf7* (forward 5'-CACGACACCGGCTACTTCTCA-3', reverse 5'-CACTCGCATCCTTCCCATG-3'), *p21*$^{waf/cip}$ (forward 5'-CCACAGCGATATCCAGACATT-3', reverse 5'-GCGGAACAGGTCGGACAT-3'), microtubule-associated protein 2 (*Map2*) forward 5'-AACGGGATCAACGGAGAGCT-3', reverse 5'-TGTGACTACTTGAACTATCCTTGCAGAT-3'), tyrosine kinase receptor A (*TrkA*) (forward 5'-TCGTGGCTGTCAAGGCACT-3', reverse 5'-CAGCTCGGCCTCACGCT-3'), *Vgf* (forward 5'-GGCTCGAATGTCCGAAAGC-3', reverse 5'-GACACTCCTTCCCCGAACTG-3'), *Scg10* (forward 5'-TGCAGAGGAGCGAAGAAAGTCT-3', reverse 5'-GCTCCCTCTTCTCTGCCAACT-3'), *βIII tubulin* (forward 5'-CGTGGGCTCAAAATGTCATC-3', reverse 5'-TGGCTGTGAACTGCTCCGAGAT-3'), *Gfap* (forward 5'-GAGGGACAACTTTGCACAGGA-3', reverse 5'-CCAGCCTCAGGTTGGTTTCAT-3'), *Ng2* (forward 5'-GCTTCCACTTCGACCTCTCTGA-3', reverse 5'-CTGAGCGGCTGCACAGACT-3'), *Ki67* (forward 5'-ATTGACCGCTCCTTTAGGTATGAA-3', reverse 5'-

GGTATCTTGACCTTCCCCATCA-3').

Western blot analysis

Western blots were performed as previously described [21]. The following primary antibodies were used: mouse anti-KLF7 (1:5000, Abnova, Taipei, Taiwan), rabbit anti-brain lipid-binding protein/ fatty acid-binding protein 7 (BLBP/ FABP7) (1:1000, Abcam, Cambridge, MA), mouse anti-TRKA (1:500, Calbiochem, Milan, Italy) or mouse anti-βACTIN (1:5000), Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Immunocytochemical analysis

Immunocytochemical analysis was performed as previously described [20]. The following primary antibodies were used: mouse anti-βIII tubulin (1:1000, Promega, Madison, WI), mouse anti-glial fibtrillary acidic protein (GFAP) (1:400, Sigma-Aldrich), rabbit anti-NG2 (1:400, Chemicon), rabbit anti-BLBP/ FABP7 (1:500), mouse anti-Ki67 (1:300, BD Pharmigen) and rabbit anti-phospho-histone H3 (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA). The antigens were revealed with anti-mouse or anti-rabbit secondary fluoresceine or rhodamine conjugated antibodies (1:400, Chemicon). Cells counting was performed on five fields from three replicates for each condition and normalized with DAPI (1:5000, Roche) positive cells.

Statistical analysis

For all experiments, analysis of variance was carried out, followed by *post hoc* comparison (ANOVA, Scheffè F-test). A value of $p<0.05$ was considered significant. Data were expressed as mean ± SE. At least three independent replicates were used for RT-PCR and western blot quantitation. All the shown experiments were performed separately at least twice.

RESULTS

KLF7 is important for neuronal differentiation in PC12 neuroepithelial cells and ESCs.

To examine the involvement of KLF7 in proliferation and differentiation in the context of differentiating neuronal cells, we studied the rat pheocromocytoma PC12 cell line as an informative experimental model. PC12 cells proliferate when cultured in the presence of serum; upon NGF stimulation, they exit the cell cycle and differentiate toward a sympathetic neuronal phenotype [22]. We first analyzed *Klf7* expression during PC12 cells differentiation using quantitative qRT-PCR. RNA analysis showed a significant increment in *Klf7* transcript accumulation after seven days of NGF-induced neuronal differentiation (Fig.

1A). This change paralleled a similar increase in *p21*$^{waf/cip}$ expression (Fig. 1A), as previously shown [3].

To assess the role of *Klf7* we first down–regulated its expression by an RNAi approach using the appropriate construct from an shRNA library. Levels of *Klf7* transcripts and KLF7 protein (84% decrease) were strongly reduced in proliferating cells stably transfected with the construct expressing a *Klf7* shRNA but not with a non-silencing control construct (Fig. 1B and 1C). *Klf7* silencing was associated with a significant down-regulation of the NGF receptor *TrkA* transcripts and protein (Fig. 1B and 1C). This result was consistent with previous *in vivo* and *in vitro* evidence [5,23], thus providing proof for the validity of using a gene silencing approach to characterize the functions of KLF7. In addition, we observed a significant decrease in transcripts of the neuronal dendritic marker MAP2 in PC12 cells stably transfected with the sh*Klf7* construct (Fig. 1B). Since there was a positive correlation between neuronal differentiation and *Klf7* gene expression in PC12 cells, we next examined the effect on differentiation associated with *Klf7* gene silencing. Our study showed that induction of neuronal differentiation by NGF treatment in *Klf7*-silenced PC12 cells resulted in a strong decrease of neurite outgrowth compared to controls (Fig. 1D). This finding is consistent with the demonstration that *Klf7*-silenced PC12 cells lack *TrkA* and, consequently, its downstream signaling mechanisms, necessary for PC12 cell neuronal differentiation [24]. To validate these findings we analyzed NGF-dependent gene expression. The results show that both *Vgf* and *Scg10* transcripts

were less expressed as compared to PC12 stimulated control cells (Fig. 2A and 2B). Moreover since PC12 differentiation is paralleled by cell cycle exit we also evaluated the expression of $p21^{waf/cip}$ cell cycle regulator. We found that in *Klf7* silenced PC12 cells, $p21^{waf/cip}$ was strongly affected as compared to controls (Fig. 2C). These results suggest further that KLF7 is involved in neuronal differentiation by contributing to the expression/ maintenance of specific neuronal genes.

To investigate further the role of KLF7 in neuronal differentiation, we utilized the pluripotent ESC line E14Tg2A, which can be induced to differentiate into neurons by serum deprivation. Following a first decrease of *Klf7* transcripts at 2d, when compared to the time of serum withdrawal (0 d), there was a significant increase in *Klf7* gene expression during E14Tg2A neuronal differentiation, which reaches a plateau at 10d (Fig. 3A). We then performed *Klf7* gene silencing experiments. We first showed in Fig. 3B a good efficiency of KLF7 down-regulation by shRNA in undifferentiated ESCs. Subsequently we analyzed morphological phenotype showing, a marked delay (2 days) of neurite formation in differentiating E14Tg2A cells stably transfected with sh*Klf7* compared to cells harbouring the control plasmid (Fig. 3C). At day 10 following differentiation neurite formation recovers in *Klf7*-silenced ESCs and becomes comparable to the control samples (data not shown). Finally, to assess the involvement of KLF7 in the transition from proliferation to the differentiation, we carried out a molecular analysis of proliferation and neural markers in

control and *Klf7*-silenced ESCs during differentiation. With regard to the proliferation markers, in *Klf7*-silenced ESCs, we found a transient downregulation of $p21^{waf/cip}$ between days 0 and 4 and an increase of *Ki67* mRNA at day 0. Neural differentiation was assessed by analyzing the expression of neuronal (*βIII tubulin*) glial (*Gfap*) and oligodendroglial (*Ng2*) markers. We found that only *βIII tubulin* transcripts were transiently decreased in *Klf7*-silenced samples (Fig. 4). Taken together these findings provide evidence that KLF7 is important for neuronal differentiation in cultured cells.

Characterization of neural stem cells (NSCs) generated from *Klf7* -/- mice.

To further study the role of KLF7 role in neuronal differentiation we used primary neurospheres from NSCs from *Klf7*-/ - mice. As shown previously [17,25], this experimental model is suitable to study how proliferating neural progenitors exit cell cycle and differentiate into the neural cell lineages. Neurospheres were generated from the SGZ of newborn *Klf7* -/ - or control mice and NSCs were induced to differentiate into the three neural lineages, neurons, astrocytes and oligodendrocytes as described previously [17]. Immunocytochemical analysis showed that KLF7 deficiency did not prevent the appearance of neuronal (βIII tubulin), glial (GFAP) or oligodendroglial (NG2) markers following induction of neural differentiation (data not shown).

We next asked if KLF7 ablation could affect NSCs proliferation and self-

renewal. To this end, we analyzed markers such as Ki67, which is expressed in almost all mitotically active cells, and phospho-histone H3, which marks only cells in M phase of the cell cycle. Ki67 and phospho-histone H3 immunostaining did not show detectable differences between wt and *Klf7* -/ - samples (data not shown). However, the BLBP/ FABP7 protein, a marker of undifferentiated mitotic neurogenic astrocytes [26], was expressed in fewer cells in *Klf7* -/ - NSCs compared to wt cells (Fig. 5A and 5B). This finding was confirmed by western blot analysis in both proliferating and differentiated NSCs (Fig. 5C). Finally, to evaluate KLF7 role in NSCs self-renewal we performed a neurosphere assay. The latter did not show any significant differences in the size and number of neurospheres between wt and *Klf7* -/ - samples (data not shown). Taken together, the results of the neurosphere assays suggest that KLF7 is dispensable for NSCs self-renewal but is necessary to maintain at least some of the molecular features characteristic of the undifferentiated neural state, such as BLBP/ FABP7 expression.

KLF7 depletion alters mesodermal cell lineages differentiation.

Recent data has suggested that KLF7 might play a wider role during development and differentiation, in addition to neuroectodermal tissues [6,7]. In particular, KLF7 might be involved in the differentiation of mesodermal cell lineages. To assess the role of KLF7 in mesodermal differentiation, we examined its involvement in the cardiomyogenesis potential of ESCs. Our studies showed

that during the differentiation of ESCs along the cardiomyocytic lineage, there was a significant up-regulation of *Klf7* transcripts (Fig. 6A). More interestingly, we observed a dramatic decrease in the number of beating cardiomyocytes derived from sh*Klf7*-ESCs when compared to control cells at day 8. We did not observe a delay in the appearance of beating activity in sh*Klf7*-ESCs compared to the control. We did not observe further increase in the number of beating embryoid bodies derived from sh*Klf7*-ESCs at day 10 or later (Fig. 6B). These results point towards a role for KLF7 in cardiomyogenic differentiation.

In order to increase our understanding of KLF7 role along other mesodermal lineages, we generated MEFs from *Klf7* -/ - mice and set up *ad hoc* protocols to induce the osteogenic and adipogenic differentiation of these cells. Following adipogenic induction, *Klf7* -/ - MEFs exhibited a significantly lower adipogenic differentiation ability compared to wt MEFs (Fig. 6C). Conversely, *Klf7* -/ - MEFs grown in osteogenic medium, were more effective at producing a well mineralized matrix (Fig. 6D), suggesting an increase in the osteogenic differentiation ability of *Klf7* -/ - compared to wt MEFs.

In summary these findings, skematized in Fig. 7, implicate KLF7 in cardiomyocytic, adipocytic and osteocytic as well as neuronal differentiation. Moreover, they show that KLF7 depletion may exert either positive or negative effects on these differentiation processes.

Oct4 and _Nanog_ gene silencing results in transcriptional up-regulation of _Klf7_.

The results described above highlight roles for KLF7 ranging from neuroectodermal to mesodermal lineages, raising the possibility that KLF7 plays roles at the level of multipotent progenitor cells. Consistent with this hypothesis, a number of KLF-family members have been involved in ESCs self-renewal pathways [2]. It has beeen suggested that, *Klf7* could be a potential target of ESCs self-renewal master genes, such as *Oct4* and *Nanog* [9,27]. Based on these observations, we next examined whether *Klf7* expression was affected by modulation of *Oct4* and *Nanog* gene expression in ESCs. To this end, we generated ESCs stable clones silenced for *Oct4* or *Nanog* genes, using specific shRNA constructs, and analyzed *Klf7* transcripts level by qRT-PCR. This analysis did not show any significant difference in undifferentiated/ proliferating cells (Fig. 8A), possibly because *Klf7* transcript levels are too low under these culture conditions. Therefore, we analyzed *Klf7* transcripts level after 13 days following induction of ESCs differentiation by serum deprivation, a process that results in increased *Klf7* expression (Fig. 8A). The analysis of sh*Oct4*- or sh*Nanog*-ESC stable clones showed a significant further increase of *Klf7* in the *Oct4*-silenced cells compared to control cells. A similar, albeit more modest, increase of *Klf7* transcripts was also observed in *Nanog*-silenced ESCs (Fig. 8A). Moreover we analyzed *Klf7* transcripts upon overexpression of either *Oct4* or *Nanog* genes in ESCs. The results of the qRT-PCR showed a significant decrease of *Klf7* expression (Fig. 8B). These results suggest that *Oct4*, and possibly *Nanog*, may

exert a negative regulation upon *Klf7* transcription. Thus, like other differentiation genes, *Klf7* could possibly be switched off in proliferating ESCs, a mechanism widely employed by ESCs to maintain self-renewal ability [27,28].

DISCUSSION

In this paper, we have used a loss of function approach in a variety of *in vitro* experimental model systems to investigate the role of the transcription factor KLF7 in cell differentiation. During embryogenesis, *Klf7* is predominantly expressed in the developing nervous system, where it plays important roles in neurogenesis [3,4]. Therefore, we first focused our attention on KLF7 role in neuronal differentiation. Using an RNAi-mediated knockdown approach in the neuroepithelial cell line PC12, we observed that down-regulation of *Klf7* gene expression causes a substantial silencing of genes crucial for neuronal differentiation, namely *Map2* and the high affinity receptor for NGF, *TrkA* [29-31]. As a consequence of *TrkA* down-regulation, sh*Klf7*-PC12 stable clones could not be differentiated into neurons, as expected in these conditions [32]. This finding corroborates in a neuronal cell line previous data demonstrating that KLF7 activates *TrkA* transcription and that TRKA is strongly reduced in *Klf7* -/ - mice [5,23]. It is worth noting that the silencing of *Klf7* in PC12 cells not only

decreases TRKA protein expression, but also dowregulates NGF-dependent signaling as showed by *Vgf* and *Scg10* expression. The latter are two genes well-known to be altered when NGF signaling is affected [33,34].

To delve deeper into KLF7 role during neuronal differentiation, we used two additional well established cellular systems, ESCs and NSCs, both of which allow the study of differentiation processes. We demonstrated a delayed neuronal differentiation in *Klf7*-silenced ESCs, indicating that the activity of this transcription factor is important for the correct timing of this process, as monitored by neurite outgrowth. A similar effect was observed after deletion of the transcription factors, WNT7A, SOX10 and MASH1 [35-37], suggesting that different mechanisms are involved in the correct timing of neuritogenesis. We did not observe any difference in neurite formation between wt and *Klf7* -/ - NSCs. *Klf7* expression differs throughout mouse embryonic development [3] and is reduced at birth. Since NSCs used for these experiments were obtained from newborn mice, a likely explanation lies upon a different role exerted by KLF7 during early phase of development versus newborn stage.

Interestingly, KLF7 down-regulation did not affect differentiation of NSCs into its three canonical lineages, as indicated by the expression of major neuronal, astrocytic or oligodendrocytic markers. However *Klf7* -/ - neurospheres displayed a BLBP/ FABP7 protein deficit compared to controls. These data are consistent with previous findings that *Blbp/Fabp7* -/ - mice display normal brain differentiation as observed by morphological and immunohistological analyses

[38]. Thus, KLF7 may act as a regulator of BLBP/ FABP7 expression, at least under our experimental conditions. To the best of our knowledge this is the first evidence linking a member of KLF family to the expression of BLBP/ FABP7. The latter is specifically expressed in neuronal progenitors in the developing CNS [14]. Therefore, although we did not find major alterations in neural phenotypes differentiated from *Klf7* -/ - NSCs, our finding show that the number of cells expressing BLBP/ FABP7is decreased suggests that a subtle alteration in the phenotypic differentiation may occur in the absence of KLF7. A deeper and more extensive analysis is needed to resolve this issue.

We hypothesized that KLF7 role in cellular differentiation was not limited to the NS. Therefore, we tested whether KLF7 would be involved in the differentiation of various mesodermal lineages. Our studies showed that *Klf7*-silenced ESCs are strongly impaired in their ability to differentiate into cardiomyocytes, and that *Klf7* -/ - MEFs display impaired adipogenesis and enhanced osteogenesis ability. In MEFs, *Klf7* depletion may alter the balance between adipogenesis and osteogenesis, as reported by others for other transcription and growth factors such as PPARγ [39], TAZ [40] and WNT family members [41,42]. It should be emphasized that our finding that *Klf7* -/ - MEFs display impaired adipogenesis is in contrast with the findings of other groups [6,7] who showed that *Klf7* overexpression in preadipocytes inhibits adipogenesis. An explanation might lie on the different experimental system used.

Overall, KLF7 appears to regulate neuroectodermal or mesodermal differentiation pathways in a manners depending on specific cellular contexts. In ESCs, *Oct4* and *Nanog* silencing increases *Klf7* gene expression. Inhibition of gene expression by OCT4 and NANOG has also been reported for other genes involved in ESCs differentiation, such as *Dkk1*, *Cdx2*, *Gata4*, *Mash1*, *Cxcl12* and *Pax3* [8,9]. Similarly, KLF6, the KLF-family member most related to KLF7 [43], is also up-regulated following NANOG down-regulation in ESCs [8]. Moreover either *Oct4* or *Nanog* overexpression leads to *Klf7* transcripts down-regulation. On the other hand Ivanova et al., showed that *Klf7* overexpression results in ESCs differntiation, loss of alkaline phosphatase activity and Nong down-regulation. Taken together these data point towards a role of KLF7 in ESCs proliferation and differentiation mechanisms. In conclusion, this work provides evidence suggesting that KLF7 is involved in multiple cell lineage differentiation mechanisms.

Acknowledgments

We wish to thank Prof. Tommaso Russo (CEINGE, Naples) for providing some reagents, Simona Ciriello (CEINGE, Naples) for technical help; Dr. Gian Carlo Bellenchi (IGB) and Prof. Carla Perrone-Capano (Univ FedericoII) for reading the manuscript.

This work was supported by MIUR PRIN 2007F7AJYJ_004 and PRIN 2007STRNHK_002, by Italian Ministry of Health Young-investigator project "under-40

call 2007".

S.S. was supported by funds from the Canadian Institutes of Health Research (MOP-13957) and by a Chercheur National Award from the Fonds de la Recherche en Sante du Quebec.

REFERENCES

[1] J.J. Bieker, Kruppel-like factors: three fingers in many pies, J. Biol. Chem. 276 (2001) 34355-34358.

[2] J. Jiang, Y.S. Chan, Y.H. Loh, J. Cai, G.Q. Tong, C.A. Lim, P. Robson, S. Zhong, H.H. Ng, A core Klf circuitry regulates self-renewal of embryonic stem cells, Nat. Cell Biol. 10 (2008) 353-360.

[3] F. Laub, R. Aldabe, V. Jr. Friedrich, S. Ohnishi, T. Yoshida, F. Ramirez, Developmental expression of mouse Kruppel-like transcription factor KLF7 suggests a potential role in neurogenesis, Dev. Biol. 233 (2001) 305-318.

[4] F. Laub, L. Lei, H. Sumiyoshi, D. Kajimura, C. Dragomir, S. Smaldone, A.C. Puche, T.J. Petros, C. Mason, L.F. Parada, F. Ramirez, Transcription factor KLF7 is important for neuronal morphogenesis in selected regions of the nervous system, Mol. Cell Biol. 25 (2005) 5699-5711.

[5] L. Lei, F. Laub, M. Lush, M. Romero, J. Zhou, B. Luikart, L. Klesse, F. Ramirez, L.F. Parada, The zinc finger transcription factor Klf7 is required for TrkA gene expression and development of nociceptive sensory neurons, Genes Dev. 19 (2005) 1354-1364.

[6] A. Kanazawa, Y. Kawamura, A. Sekine, A. Iida, T. Tsunoda, A. Kashiwagi, Y. Tanaka, T. Babazono, M. Matsuda, K. Kawai, T. Iiizumi, T. Fujioka, M. Imanishi, M. Kaku, Y. Iwamoto, R. Kawamori, R. Kikkawa, Y. Nakamura, S. Maeda, Single nucleotide polymorphisms in the gene encoding Kruppel-like factor 7 are associated with type 2 diabetes, Diabetologia 48 (2005) 1315-1322.

[7] Y. Kawamura, Y. Tanaka, R. Kawamori, S. Maeda, Overexpression of Kruppel-like factor 7 regulates adipocytokine gene expressions in human adipocytes and inhibits glucose-induced insulin secretion in pancreatic beta-cell line, Mol. Endocrinol. 20 (2006) 844-856.

[8] Y.H. Loh, Q. Wu, J.L. Chew, V.B. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K.Y. Wong, K.W. Sung, C.W. Lee, X.D. Zhao, K.P. Chiu, L. Lipovich, V.A. Kuznetsov, P. Robson, L.W. Stanton, C.L. Wei, Y. Ruan, B. Lim, H.H. Ng, The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells, Nat. Genet. 38 (2006) 431-440.

[9] N. Ivanova, R. Dobrin, R. Lu, I. Kotenko, J. Levorse, C. DeCoste, X. Schafer, Y. Lun, I.R. Lemischka, Dissecting self-renewal in stem cells with RNA interference, Nature 442 (2006) 533-588.

[10] H. Kawasaki, K. Mizuseki, S. Nishikawa, S. Kaneko, Y. Kuwana, S. Nakanishi, S.I. Nishikawa, Y. Sasai, Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity, Neuron 28 (2000) 31-40.

[11] Q.L. Ying, M. Stavridis, D. Griffiths, M. Li, A. Smith, Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture, Nat. Biotechnol. 21 (2003) 183-186.

[12] A. Fico, G. Manganelli, M. Simeone, S. Guido, G. Minchiotti, S. Filosa, High-throughput screening-compatible single-step protocol to differentiate embryonic stem cells in neurons, Stem Cells Dev. 17 (2008) 573-584.

[13] S. Parisi, F. Passaro, L. Aloia, I. Manabe, R. Nagai, L. Pastore, T. Russo, Klf5 is involved in self-renewal of mouse embryonic stem cells, J. Cell Sci. 121 (2008) 2629-2634.

[14] V.A. Maltsev, J. Rohwedel, J. Hescheler, A.M. Wobus, Embryonic stem cells

differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types, Mech. Dev. 44 (1993) 41-50.

[15] N. Sherwood, S.P. Timiras, A stereotaxic atlas of the developing rat brain, second ed., University of California press, Berkeley, 1970.

[16] L. Kruger, S. Saporta, L.W. Swanson, Photographic atlas of the rat brain: The cell and fiber architecture illustrated in three planes with stereotaxic coordinates, third ed., Cambridge University Press, Cambridge, 1995.

[17] B.A. Reynolds, S. Weiss, Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell, Dev. Biol. 175 (1996) 1-13.

[18] D.L. Alexander, L.G. Ganem, P. Fernandez-Salguero, F. Gonzalez, C.R. Jefcoate, Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis, J. Cell. Sci. 111 (1998) 3311–3322.

[19] C.M. Digirolamo, D. Stokes, D. Colter, D.G. Phinney, R. Class, D.J. Prockop, Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate, Br. J. Haematol. 107 (1999) 275-281.

[20] F. Volpicelli, C. Consales, M. Caiazzo, L. Colucci-D'amato, C. Perrone-Capano, U. di Porzio, Enhancement of dopaminergic differentiation in proliferating midbrain neuroblasts by sonic hedgehog and ascorbic acid, Neural Plast. 11 (2004) 45-57.

[21] F. Volpicelli, M. Caiazzo, D. Greco, C. Consales, L. Leone, C. Perrone-Capano, L. Colucci-D'Amato, U. di Porzio, Bdnf gene is a downstream target of Nurr1 transcription factor in rat midbrain neurons in vitro, J. Neurochem. 102 (2007) 441-453.

[22] L.A. Greene, A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheocromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 2424-2428.

[23] L. Lei, L. M, S. Nef, T. Thai, L.F. Parada, mKlf7, a potential transcriptional regulator of TrkA nerve growth factor receptor expression in sensory and sympathetic neurons, Development 128 (2001) 1147-1158.

[24] R.A. Segal, M.E. Greenberg, Intracellular signaling pathways activated by neurotrophic factors, Annu. Rev. Neurosci. 19 (1996) 463-489.

[25] C.Q. Doe, Neural stem cells: balancing self-renewal with differentiation. Development 135 (2008) 1575-1587.

[26] T.E. Anthony, C. Klein, G. Fishell, N. Heintz, Radial glia serve as neuronal progenitors in all regions of the central nervous system, Neuron 41 (2004) 881-890.

[27] M. Boiani, H.R. Scholer, Regulatory networks in embryo-derived pluripotent stem cells, Nat. Rev. Mol. Cell. Biol. 6 (2005) 872-884.

[28] K. Mitsui, Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda, S. Yamanaka, The homeoprotein Nanog is required for maintenance of plurypotency in mouse epiblast and ES cells, Cell 113 (2003) 631-642.

[29] I. Fischer, C. Ritcher-Landsberg, R. Safaei, Regulation of microtubule associated protein 2 (MAP2) expression by nerve growth factor in PC12 cells, Exp. Cell. Res. 194 (1991) 195-201.

[30] L. Dehmelt, S. Halpain, The MAP2/ Tau family of microtubule-associated proteins, Genome Biol. 6 (2005) 204.1-204.10.

[31] M.V. Chao, B.L. Hempstead, p75 and Trk: a two-receptor system, Trends Neurosci. 18 (1995) 321-326.

[32] D.M. Loeb, J. Maragos, D. Martin-Zanca, M.V. Chao, L.F. Parada L.A. Greene, The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines, Cell 66 (1991) 961-966.

[33] D. Califano, A. D'Alessio, G.L. Colucci-D'Amato, G. De Vita, C. Monaco, G. Santelli, P.P. Di Fiore, G. Vecchio, A. Fusco, M. Santoro, V. de Franciscis, A potential pathogenetic mechanism for multiple endocrine neoplasia type 2 syndromes involves ret-induced impairment of terminal differentiation of neuroepithelial cells, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7933-7937.

[34] G.L. Colucci D'Amato, A. D'Alessio, D. Califano, G. Cali, C. Rizzo, L. Nitsch, G. Santelli, V. de Franciscis, Abrogation of nerve growth factor-induced terminal differentiation by ret oncogene involves perturbation of nuclear translocation of ERK, J. Biol. Chem. 275 (2000) 19306-19314.

[35] A.C. Hall, F.R. Lucas, P.C. Salinas, Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling, Cell 100 (2000) 525-535.

[36] J. Kim, E. Dormand, D.J. Anderson, SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells, Neuron 38 (2003) 17-31.

[37] A. Pattyn, F. Guillemot, J.F. Brunet, Delays in neuronal differentiation in Mash1/ Ascl1 mutants, Dev. Biol. 295 (2006) 67-75.

[38] Y. Owada, S.A. Abdelwahab, N. Kitanaka, H. Sakagami, H. Takano, Y. Sugitani, M. Sugawara, H. Kawashima, Y. Kiso, J.I. Mobarakeh, K. Yanai, K. Kaneko, H. Sasaki, H. Kato, S. Saino-Saito, N. Matsumoto, N. Akaike, T. Noda, H. Kondo, Altered emotional behavioural responses in mice lacking brain-type fatty acid-binding protein gene, Eur. J. Neurosci. 24 (2006) 175-187.

[39] E.D. Rosen, Adipocyte differentiation from inside out, Nat. Rev. Mol. Cell. Biol. 7 (2006) 885-896.

[40] J.H. Hong, E.S. Hwang, M.T. McManus, A. Amsterdam, Y. Tian, R. Kalmukova, E. Mueller, T. Benjamin, B.M. Spiegelman, P.A. Sharp, N. Hopkins, M.B. Yaffe, TAZ, a transcriptional modulator of mesenchymal stem cell differentiation, Science 309 (2005) 1074-1078.

[41] J. Liu, S.R. Farmer, Regulating the balance between peroxisome proliferator-

activated receptor gamma and beta-catenin signaling during adipogenesis. A glycogen synthase kinase 3beta phosphorylation-defective mutant of beta-catenin inhibits expression of a subset of adipogenic genes, J. Biol. Chem. 279 (2004) 45020-45027.

[42] C. Hartmann, A Wnt canon orchestrating osteoblastogenesis, Trends Cell Biol. 16 (2006) 151-158.

[43] N. Matsumoto, F. Laub, R. Aldabe, W. Zhang, F. Ramirez, T. Yoshida, M. Terada, Cloning the cDNA for a new human zinc finger protein defines a group of closely related Kruppel-like transcription factors, J. Biol. Chem. 273 (1998) 28229-28237.

FIGURE LEGENDS

Fig. 1 KLF7 is involved in PC12 cells differentiation.

A) *Klf7* and *p21*$^{waf/cip}$ transcripts show a remarkable increase in PC12 cells upon nerve growth factor (NGF) stimulation (+NGF) when compared to untreated cells (-NGF). **B**) When *Klf7* gene is silenced in proliferating PC12 cells a severe

down-regulation of *TrkA*, *Map2* and *Klf7* transcripts is observed compared to controls. C) Western blot analysis in proliferating PC12 cells shows that KLF7 and TRKA protein levels are highly reduced in *Klf7*-silenced (sh*Klf7*) cells compared to controls and normalized to and βACTIN. D) The pictures show the strong neurite outgrowth deficit of sh*Klf7*-PC12 cells following 7 days of NGF treatment (upper panel). The result was confirmed by total neurite length quantitation (lower panel).

Analyses were performed in PC12 cells stably transfected with control or sh*Klf7* constructs. The diagrams show the relative quantitation (mean ± SE) of the *Klf7*, $p21^{waf/cip}$, *Map2* and *TrkA* amplified products compared to that of the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, internal standard). Data are expressed as ratio *Klf7*/ *Gapdh*, $p21^{waf/cip}$/ *Gapdh*, *Map2*/ *Gapdh* and *TrkA*/ *Gapdh*. Asterisks represent $p \leq 0.01$ (**) when compared to control cultures (ANOVA, Scheffè F-test). Scale bar 100 μm.

Fig. 2 Transcriptional analysis of $p21^{waf/cip}$ and NGF-dependent expression in PC12 cells.

The transcriptional analysis shows a transient decrease of *Vgf* (A) at day 1, and a decrease of *Scg10* (B) and $p21^{waf/cip}$ (C) between day 3 and 7 of differentiation in *Klf7* silenced (sh*Klf7*) PC12 cells. The diagrams show the relative quantitation (mean ± SE) of the *Vgf* (A), *Scg10* (B), $p21^{waf/cip}$ (C) amplified products compared to

that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, internal standard). Data are expressed as ratio *Vgf*/ *Gapdh*, *Scg10/Gapdh* and $p21^{waf/cip}$/ *Gapdh*. Analyses were performed in control and sh*Klf7* PC12 cells during 7 days (d) of NGF-induced neuronal differentiation. Asterisks represent $p \leq 0.01$ (**) when compared to control cultures (ANOVA, Scheffè F-test).

Fig. 3 KLF7 is involved in ESCs neuronal differentiation.

A) *Klf7* gene expression in embryonic stem cells (ESCs) show a significant increase at 10-13 days (d), of neuronal differentiation, after an initial relative decrease. The diagrams show the relative quantitation (mean ± SE) of the *Klf7* amplified product compared to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, internal standard). Data are expressed as ratio *Klf7*/ *Gapdh*. **B**) *Klf7* gene silencing in undifferentiated ESCs. The western blot shows KLF7 and βACTIN protein levels in control and *Klf7*-silenced (sh*Klf7*) ESCs. **C**) Neurite outgrowth is delayed in sh*Klf7*-ESCs after differentiation as observed at day 8, whereas (data not shown) no differences were observed at day 10. Images represent control or *shKlf7*-ESCs after 8 days of neuronal induction. The insets are enlargement of the areas indicated by squared brackets.

Asterisks represent $p \leq 0.05$ (*) or $p \leq 0.01$ (**) (ANOVA, Scheffè F-test). Scale bar, 50 μm.

Fig. 4 Transcriptional analysis of neural differentiation and proliferation markers in ESCs.

The transcriptional analysis shows a transient decrease of *βIII tubulin* (A) at day 8 and *p21*$^{waf/cip}$ (D) between day 0 and 4 of neuronal differentiation in *Klf7*-silenced (sh*Klf7)* embryonic stem cells along (ESCs), whereas *Ki67* (E) transcripts are transiently increased at day 0. The diagrams show the relative quantitation (mean ± SE) of the *βIII tubulin* (A), *Gfap* (B), *Ng2* (C), *p21*$^{waf/cip}$ (D) and *Ki67* (E) amplified products compared to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, internal standard). Data are expressed as ratio *βIII tubulin*/ *Gapdh*, *Gfap/Gapdh*, *Ng2/Gapdh*, *p21*$^{waf/cip}$/ *Gapdh* and *Ki67*/ *Gapdh*. Analyses were performed in control and sh*Klf7* ESCs during 13 days (d) of neuronal differentiation. Asterisks represent $p \leq 0.01$ (**) when compared to control cultures (ANOVA, Scheffè F-test).

Fig. 5 The neural progenitor marker BLBP/FABP7 in *Klf7* -/- and control neural stem cells.

A) The immunocytochemical analysis shows the decrease of BLBP/ FABP7 positive (+) cells in proliferating *Klf7* -/ - neurospheres compared to wild type (wt) samples. The result was confirmed by cell counting showing the relative number of BLBP/ FABP7+ cells normalized on DAPI+ cells (**B**). **C**) The western

blot further corroborated that BLBP/ FABP7 protein decreases in *Klf7* -/ - neurospheres. The panels show BLBP/ FABP7 and βACTIN protein levels in wt and *Klf7* -/ - neurospheres in proliferating [+basic fibroblast growth factor (bFGF), +epidermal growth factor (EGF)] or growth factors deprived (-bFGF, -EGF) cultures.

BLBP/ FABP7 signal is revealed with an anti-rabbit rhodamine conjugated secondary antibody and nuclei are stained with DAPI. Asterisks represent $p \leq 0.01$ (**) when compared to wt cultures (ANOVA, Scheffè F-test). Scale bar, 100 μm.

Fig. 6 KLF7 depletion alters mesodermal cell lineages differentiation.

A) *Klf7* gene expression increases in embryonic stem cells (ESCs) and is significant between 5 and 9 days (d) of cardiomyogenic differentiation. **B**) The unique beating ability of cardiomyocytes is reduced in *Klf7*-silenced (sh*Klf7*) ESCs when compared to control ESCs. Data are expressed as percentage of beating embryoid bodies (EBs) over total EBs. **C**). Adipogenic differentiation is decreased, in *Klf7* -/ - mouse embryonic fibroblasts (MEFs) when compared to wild type (wt) cultures. The upper panels show the oil red O staining after 14 days of adipogenic induction in the two cell types. The lower panel shows the lipids content measured by absorbance at 540 nm. **D**) Osteogenic differentiation of *Klf7* -/ - MEFs is increased, when compared to wt cultures. The upper panels show the alizarin red S staining of MEFs after 21 days of osteogenic induction.

The lower panel shows calcium deposits content measured by absorbance at 490 nm.

Asterisks represent $p \leq 0.05$ (*) or $p \leq 0.01$ (**) when compared to control cultures (ANOVA, Scheffè F-test). Scale bar, 100 μm.

Fig. 7 Summary of *Klf7* silencing effect in cellular differentiation.

Klf7 silencing leads to altered differentiation in PC12 cells, mouse embryonic fibroblasts (MEFs), embryonic stem cells (ESCs) and neural stem cells (NSCs). The figure shows phase contrast images of undifferentiated PC12 cells (PC12), MEFs, ESCs or differentiated NSCs stained by βIII tubulin immunofluorescence. Arrows indicate down-regulation, up-regulation or delay (dashed arrow) of differentiation processes and markers (*Map2*, *TrkA*, BLBP/ FABP7) associated with *Klf7* silencing.

Fig. 8 *Klf7* gene expression is altered following silencing or overexpression of *Oct4* or *Nanog* .

A) *Klf7* transcripts increase in *Oct4*- or *Nanog*-silenced ESCs, following 13 days (d) of differentiation. Analyses were performed in proliferating (PROL.), or serum deprived (DIFF.) embryonic stem cells (ESCs) stably transfected with control construct (CONTROL), or *Nanog* (sh*Nanog*) or *Oct4* (sh*Oct4*) silencing

constructs. B) *Klf7* transcripts are decreased in ESCs following transient overexpression of *Oct4* (ov-*Oct4*) or *Nanog* (ov-*Nanog*) genes at 24 hours (h) and 72 h following the transfection.

The diagrams show the relative quantitation (mean ± SE) of the *Klf7* amplified product compared to that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, internal standard). Data are expressed as ratio *Klf7*/ *Gapdh*.. Asterisks represent $p \leq 0.05$ (*) or $p \leq 0.01$ (**) when compared to control cultures (ANOVA, Scheffè F-test).

"Disclosure Statement"

All authors state that there are no present or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three (3) years of beginning the work submitted that could inappropriately influence (bias) their work.

Figure 1

Click here to download high resolution image

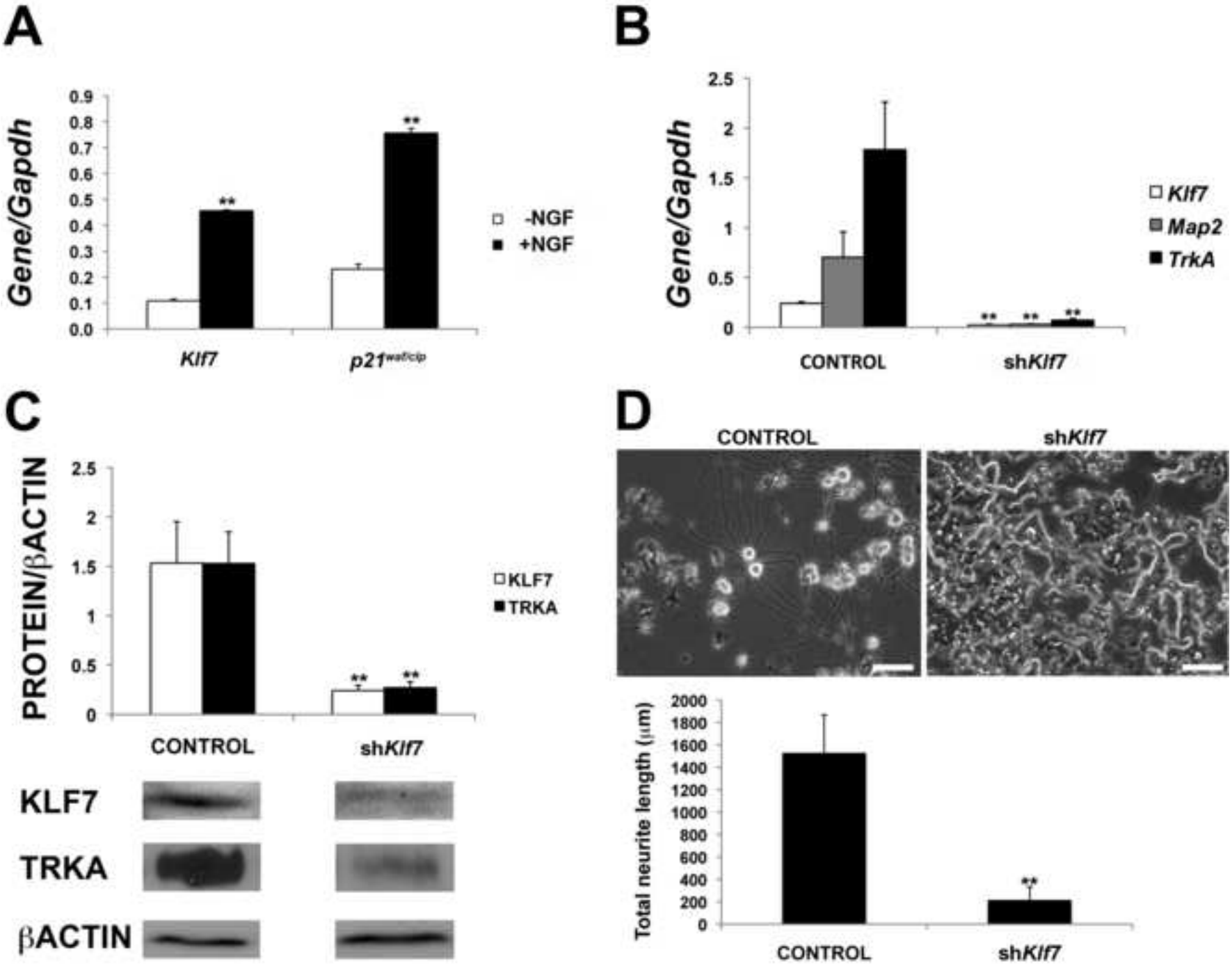

Figure 2

Click here to download high resolution image

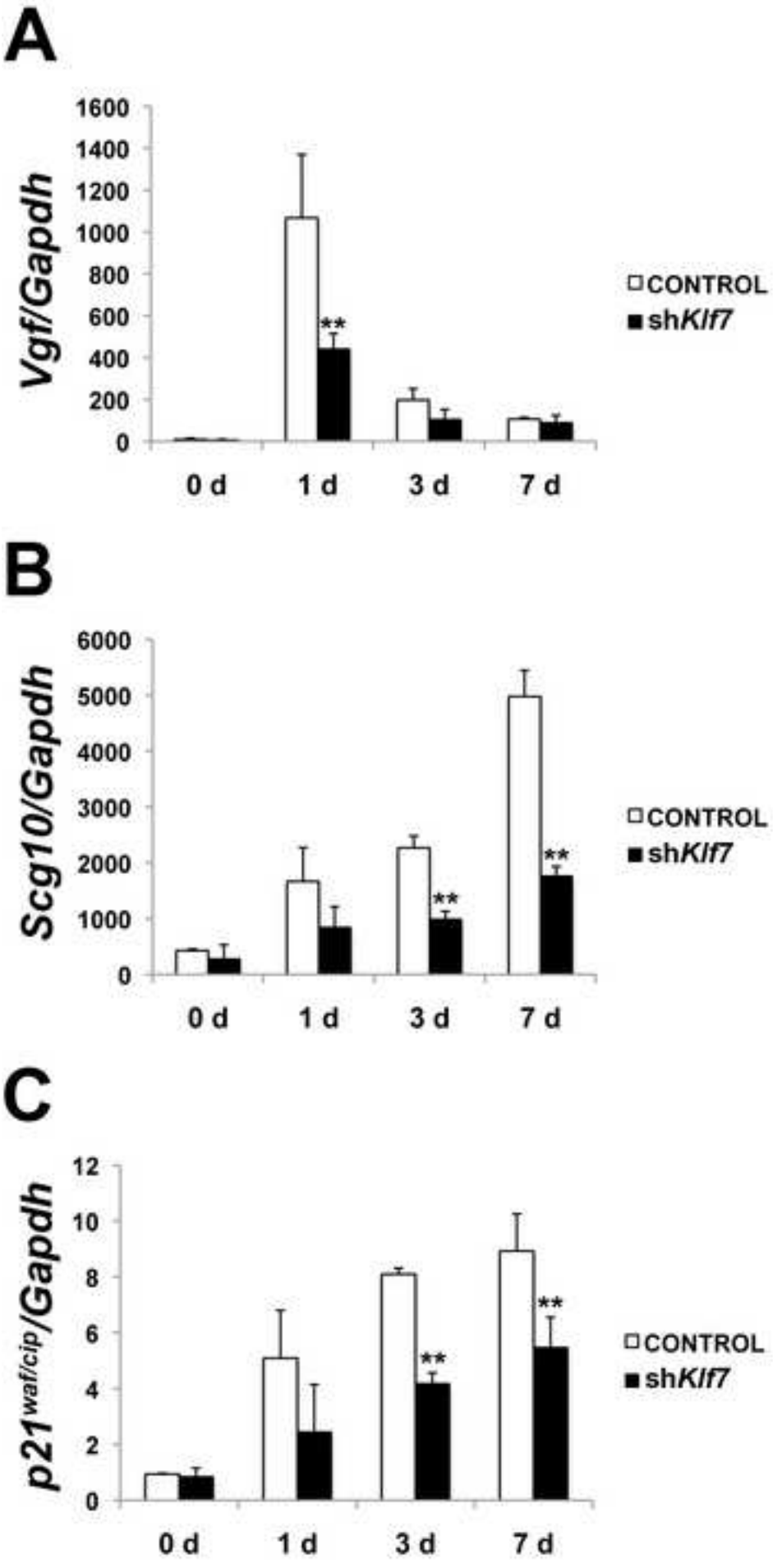

Figure 3

Click here to download high resolution image

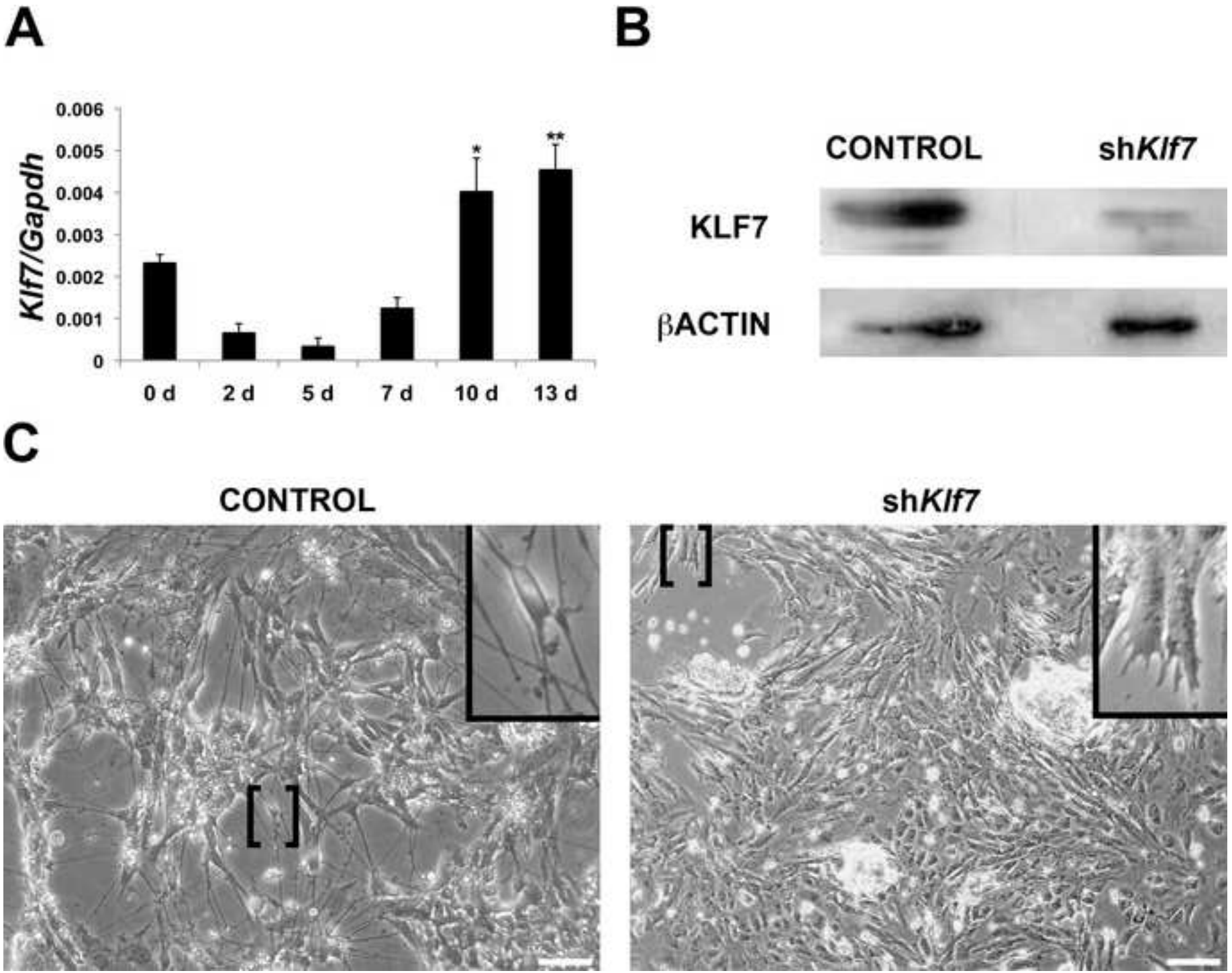

Figure 4

Click here to download high resolution image

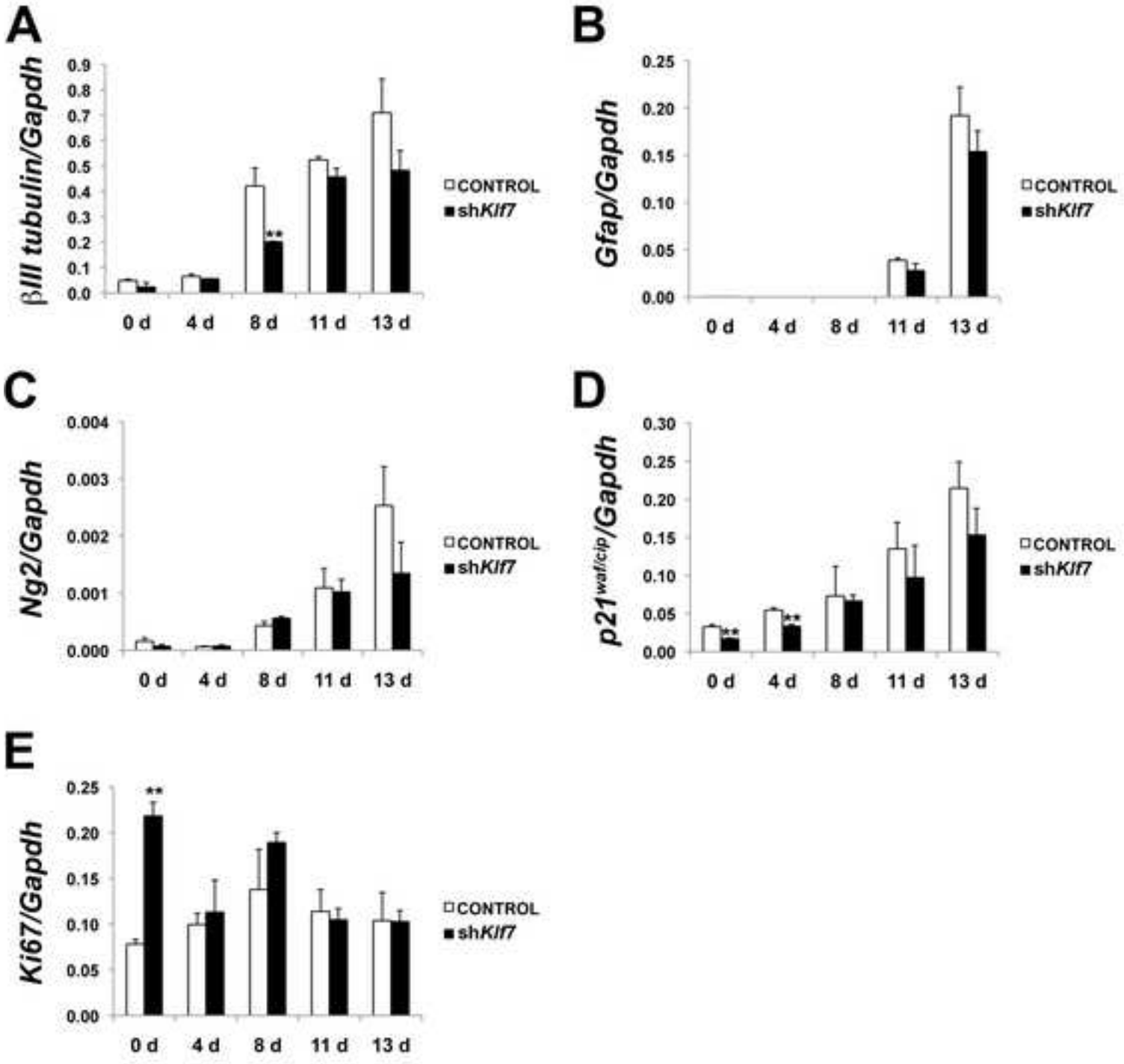

Figure 5

Click here to download high resolution image

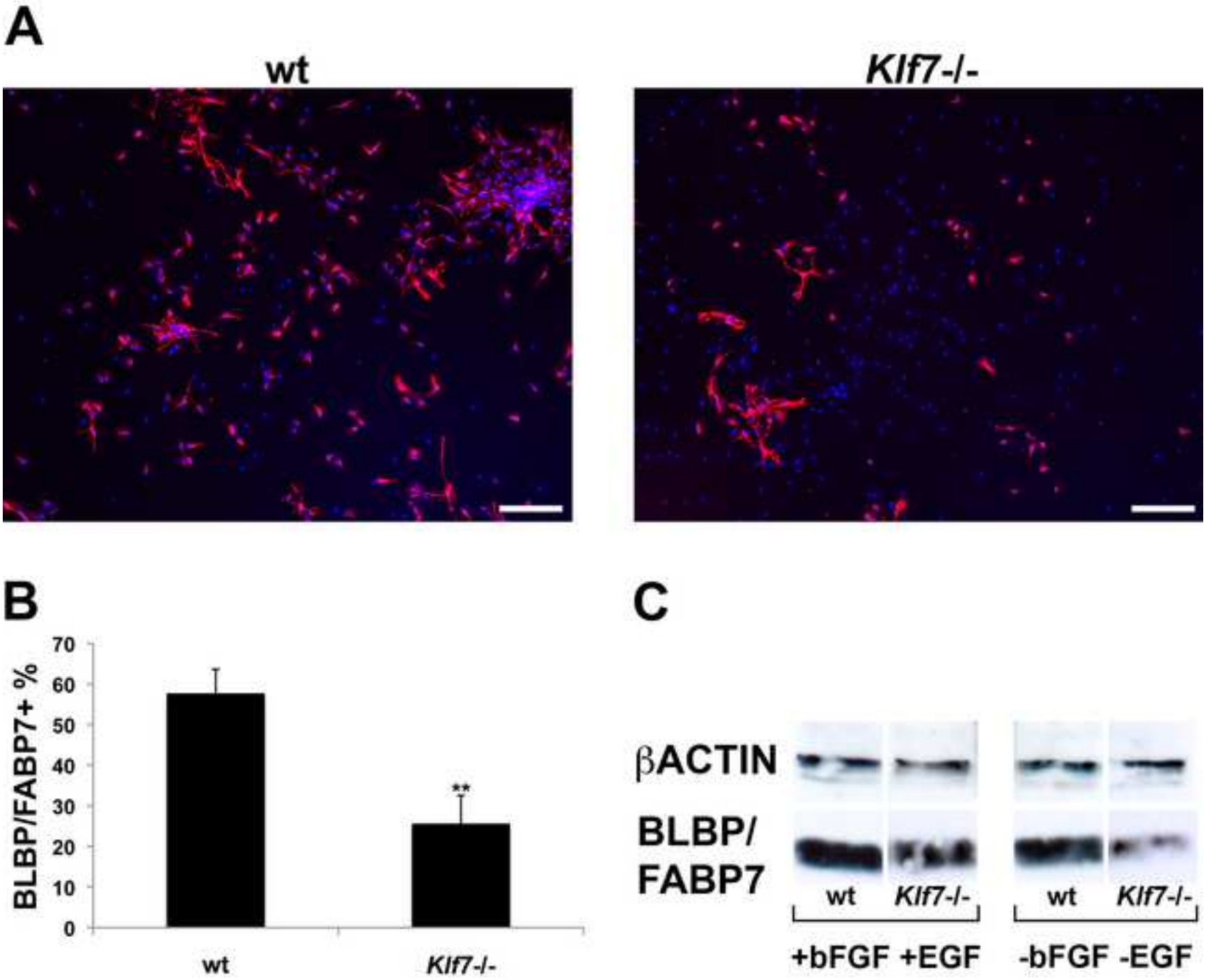

Figure 6

Click here to download high resolution image

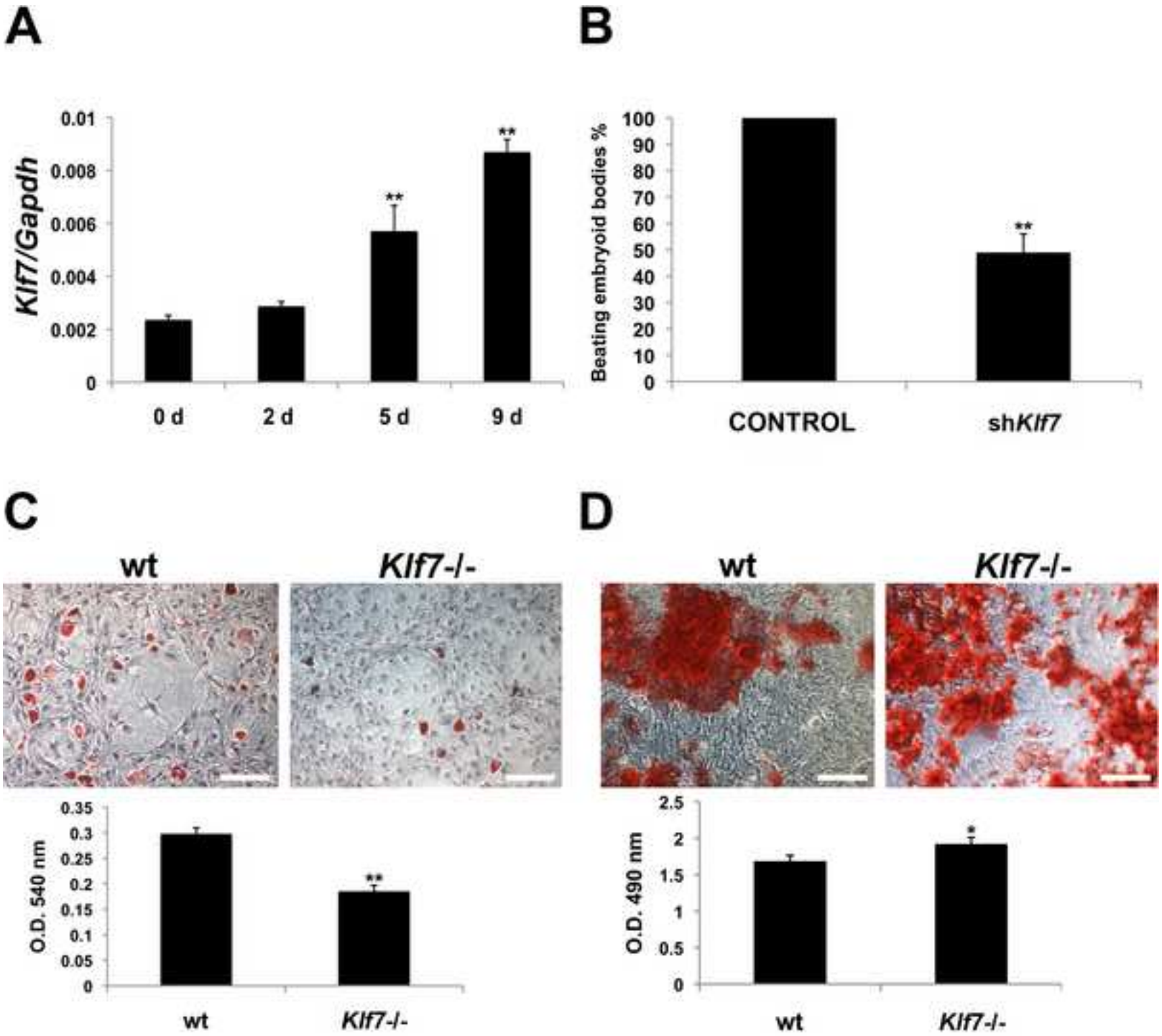

Figure 7

Click here to download high resolution image

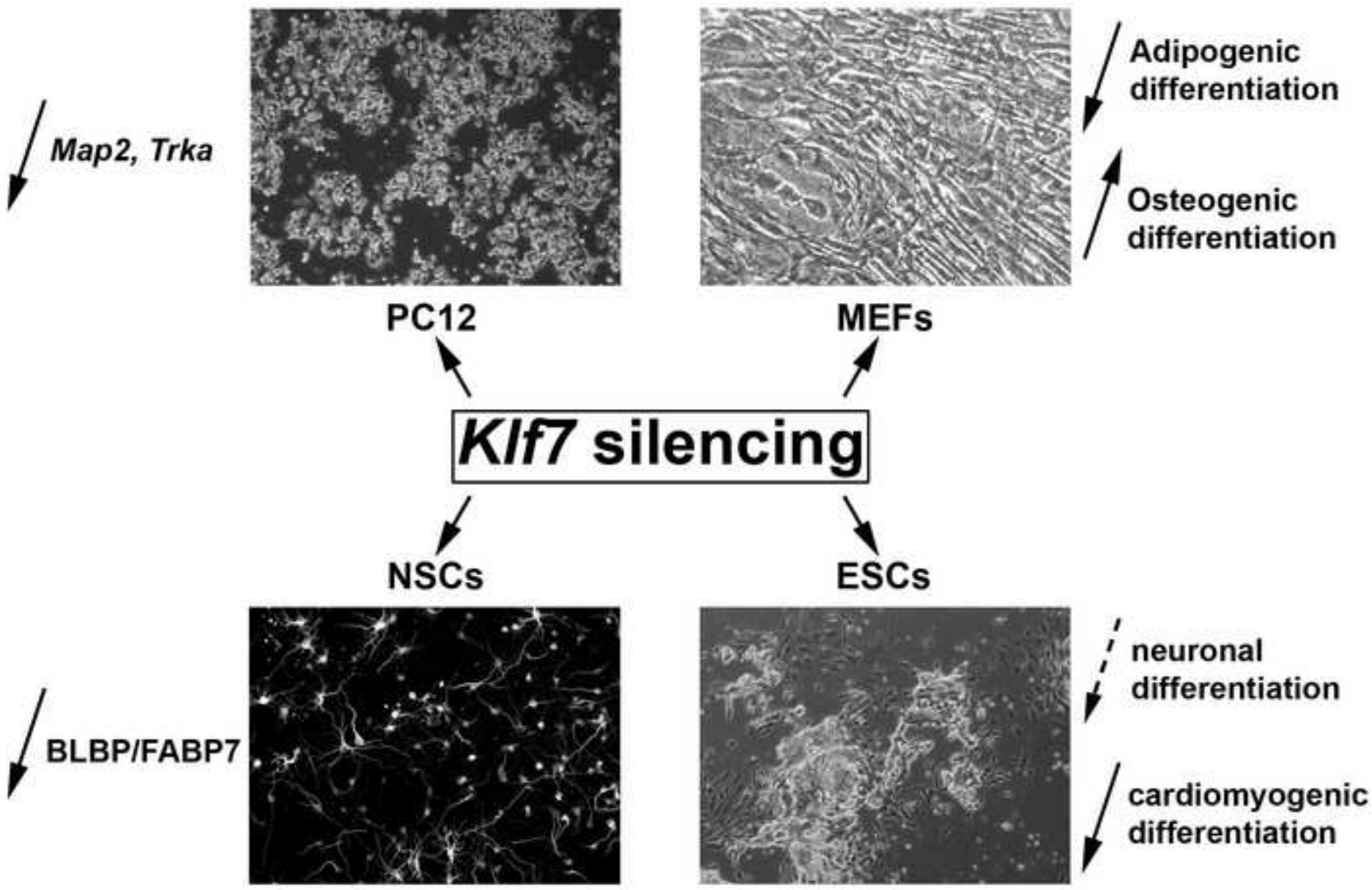

Figure 8

Click here to download high resolution image